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14. ABSTRACT

TSC1 and TSC2 are tumor suppressor genes that are mutated in tuberous sclerosis complex (TSC). Mutation in either TSC1 or TSC2 results in a constitutively activation of mammalian target of rapamycin (mTOR), therefore promoting cell growth. mTOR activity is regulated by intracellular signals, including growth factors and cellular energy level. Energy starvation, such as glucose deprivation, inhibits mTOR activity via the activation of TSC2. We have observed that TSC cells are very sensitive to cellular energy starvation. This is because TSC cells fail to stop growth even in the absence of glucose while normal cells will stop grow under glucose starvation. The energy starvation induced cell death is due to apoptosis. P53 is the most frequently mutated human tumor suppressor. It has been reported that p53 is stabilized by AMPK, which is activated by energy starvation. The major goal of this proposal is to investigate the functional importance of p53 in energy starvation induced apoptosis in TSC tumor cells. We will test whether p53 phosphorylation and protein levels are accumulated in TSC tumor cells. Furthermore, we will investigate the function of TSC1 and TSC2 in p53 regulation. Completion of this proposal will establish a functional relationship between TSC1/TSC2 and p53 tumor suppressors.

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INTRODUCTION

The mammalian target of rapamycin (mTOR) is constitutively activated in tuberous sclerosis complex tumors. mTOR is a central cell growth regulator that stimulates protein translation and increases cell size. mTOR activity is regulated by intracellular signals, including growth factors and cellular energy level. Energy starvation, such as glucose deprivation, inhibits mTOR activity via the activation of TSC2. Previous studies from our laboratory have shown that TSC cells are very sensitive to cellular energy starvation. This is because TSC cells fail to stop growth even in the low energy status while normal cells will stop grow under energy stress. The energy starvation induced cell death is due to apoptosis. P53 is the most frequently mutated human tumor suppressor. It has been reported that p53 is stabilized by AMPK, which is activated by energy starvation. The major goal of this proposal was to investigate the functional importance of p53 in energy starvation induced apoptosis in TSC tumor cells. We have shown that p53 activation is essential for energy starvation induced apoptosis in the TSC tumor cells. Furthermore, our study establishes a molecular mechanism how mTOR activation contribution to p53 accumulation and apoptosis. These results may have an important implication regarding the usage of rapamycin for future cancer treatment.

BODY

Result Summary

Dysregulation of mTOR activation sensitizes cells to p53-dependent insults

p53 is known to be activated by both energy starvation and DNA damage. To determine the effect of mTOR activation on p53 activation, TSC1-/- and TSC1+/+ MEFs were challenged with glucose starvation. TSC1-/- MEFs, which are unable to down regulate mTOR in response to low starvation, underwent massive amounts of gross cell death, as seen by the appearance of rounded floating cells. Furthermore, inhibition of mTOR by rapamycin protected the TSC1-/- MEFs against glucose starvation induced cell death. In comparison, TSC1+/+ MEFs, which properly down-regulate mTOR in response to low energy, show no evidence of cell death (Fig 1A left).

To demonstrate that p53 is important for energy starvation induced cell death during mTOR activation, p53 was knocked down in TSC1-/- MEFs by RNAi (TSC1-/- p53 RNAi MEFs). When TSC1-/- p53 RNAi MEFs are challenged with glucose starvation, they are more resistant to cell death than their control RNAi counterparts. Furthermore, rapamycin treatment of TSC1-/- p53 RNAi MEFs showed no further protection against cell death. In contrast, TSC1-/- control RNAi MEFs were acutely sensitive to glucose starvation, and mTOR inhibition was protective against glucose starvation (Fig 1A right). Together, this suggests that p53 is important for mediating the cell death seen by energy starvation in TSC1-/- MEFs.

Consistently with the fact that loss of either TSC1 or TSC2 is sufficient to induce mTOR dysregulation, TSC2-/- LEFs, derived from Eker rat kidney tumors, also show increased sensitivity to glucose starvation, which can be rescued by rapamycin treatment. Viral infection of TSC2 to restore control of mTOR also protects the LEFs from glucose starvation (Fig 1B left). These results demonstrate that downregulation of mTOR during energy starvation is necessary to prevent cell death.

Since RNAi of p53 in the TSC1-/- MEFs incompletely knocked down p53, we wanted to test the effects of mTOR activation in a p53 null background. To test whether p53 is important for regulating cellular viability in the presence of constitutive mTOR activation, TSC2-/- p53-/- and TSC2+/+ p53-/- MEFs were challenged with energy starvation. Loss of either TSC1 or TSC2 impairs the ability to down-regulate mTOR in response to glucose starvation. However,

when p53 is also missing, TSC2-/- p53-/- MEFs and TSC2+/+ p53-/- MEFs showed equal sensitivity to glucose starvation (Fig 1B right). Furthermore, rapamycin had no effect on either cell type. Therefore, loss of p53 eliminated the increased sensitivity to energy starvation induced by aberrant mTOR activation, which implies that p53 is important for mediating cell death, and mTOR may be an upstream regulator of p53.

To test whether mTOR activation also sensitized cells to other activators of p53, various cells types were used to examine the sensitivity to DNA damage. p53 is potently activated by DNA damage induced by alkylating agents such as methyl methanesulfonate (MMS) and topoisomerase inhibitors such as etoposide. Like energy starvation, MMS treatment also induced cell death in TSC1-/- MEFs, but not in WT counterparts. Furthermore, inhibition of mTOR by rapamycin pretreatment also protected against MMS induced DNA damage (Fig 1C left). Interestingly, MMS treatment inhibits mTOR activation as determined by S6K phosphorylation in TSC1+/+ and TSC2+/+ p53-/- MEFs, but not in TSC1-/- and TSC2+/+ p53-/-MEFs (Supplemental Fig 1). The role of the TSC complex in mediating mTOR inhibition by MMS was further established by TSC2 RNAi in HEK293 cells. Knockdown of TSC2 significantly compromised MMS-induced mTOR inactivation (Supplemental Fig 2). Consistently, in HEK293 cells, activation of mTOR by infection of an active mutant of Rheb (Rheb L64Q) also sensitized the cells to MMS, which was also inhibited by pre-treatment with rapamycin (Fig 1C right). Thusly, aberrant mTOR activation sensitizes cells to DNA damage.

mTOR activation enhances p53 phosphorylation and accumulation

The observations that loss of p53 reduced sensitivity to energy starvation in TSC cells (Fig 1A vs. Fig 1B) and activation of mTOR increased sensitivity to DNA damage (Fig 1C) suggested that p53 activation could be important for mediating mTOR's pro-apoptotic role. To test this possibility, p53 activation was determined by phosphorylation and accumulation. In the TSC1-/- MEFs, glucose starvation induced phosphorylation of p53 on Ser15 (mouse p53 Ser18) and accumulation of p53 protein (Fig 2A). However, the phosphorylation and accumulation of p53 could be prevented by inhibition of mTOR or AMPK with rapamycin or compound C, respectively. Rapamycin lowered p53 levels below that of basal, while compound C maintained p53 levels similar to basal. In comparison, p53 showed little activation by glucose starvation in the TSC1+/+ MEFs. However, DNA damage by etoposide activated p53 in both TSC1+/+ and TSC1-/- MEFs; therefore, it is unlikely that the TSC1+/+ MEFs are defective p53 activation (Fig 2A). Inhibition of p53 accumulation by rapamycin suggests that constitutive activation of mTOR was responsible for the difference in p53 response between the TSC1-/- and TSC1+/+ MEFs.

To verify that the p53 response was not limited to just the TSC1-/- MEFs, the TSC2-/- LEFs were also tested. Similarly, in the TSC2-/- LEFs glucose starvation induced activation of p53 as seen by phosphorylation and accumulation of p53, which was eliminated by mTOR inhibition. Consistently, add back of TSC2 also prevented p53 activation by glucose starvation (Fig 2B).

To determine whether p53 was fully activated other p53 phosphorylation sites were also analyzed by immunoblot. Ser15 phosphorylation induces dissociation between p53 and its ubiquitin E3 ligase Mdm2; however, it is insufficient to induce p53 DNA binding, which is induced by phosphorylation on p53 Ser392 (Kapoor et al, 2000). Therefore, multiple phosphorylations are necessary to fully activate p53. We show that glucose starvation induced phosphorylation on several sites including Ser6, Ser9, Ser20, and Ser392 (Fig 2C). Furthermore, phosphorylation on those sites was eliminated by the addition of rapamycin. However, the p53 protein level was also inhibited by rapamycin, therefore, it is possible p53 phosphorylation decreased indirectly by decreasing total p53 protein.

To determine whether DNA damage also induced rapamycin reversible p53

accumulation, TSC1-/- MEFs were treated with etoposide. Etoposide induced phosphorylation and accumulation of p53, and similar to glucose starvation-dependent p53 activation, rapamycin decrease the p53 protein level and the detected phosphorylation on Ser15. Moreover, treatment of TSC1-/- MEFs with rapamycin alone also decreases p53 protein levels (Fig 2D). Taken together this suggests that mTOR is a positive regulator of p53, and inhibition of mTOR attenuates p53 accumulation irrespective of the stimulus used to stabilize p53.

p53 is stabilized by energy starvation

As seen earlier, activation of p53 by energy starvation requires both AMPK activation and dysregulation of mTOR (Fig 2A). Accumulation of p53, by energy starvation could be due to either stabilization or increased synthesis of p53. To better understand the role of energy starvation on p53 activation, both p53 synthesis and degradation were examined in the TSC1-/- MEFs. To test the effect of energy starvation on p53 stability, p53 was accumulated by glucose starvation in TSC1-/- MEFs, and translation was then blocked by cycloheximide treatment. After which, the cells were maintained in either glucose-free media or switched to glucose-containing media. Restoration of glucose decreased p53 stability. This decrease in p53 stability was correlated with AMPK inactivation (Fig 3A). Similar results were also seen in the absence of cycloheximide; however, the change in p53 stability was partially masked by continued p53 synthesis (data not shown). Furthermore, glucose starvation-induced phosphorylation of p53 Ser15 was also eliminated by restoration of glucose (Fig 3A). However, the concurrent change in p53 protein level confounds conclusions about AMPK and p53 Ser15 phosphorylation with this experiment.

To test the effect of AMPK activity on p53 phosphorylation during glucose starvation, p53 was accumulated by glucose starvation in TSC1-/- MEFs, and MG132, a proteosome inhibitor, was used to prevent p53 degradation. Block of AMPK by Compound C reduced p53 Ser15 phosphorylation even in the absence of p53 degradation. This suggested that activation of AMPK was responsible for p53 Ser15 phosphorylation during energy starvation (Fig 3B).

To rule out the possibility that accumulation of p53 protein during energy starvation was due to changes in p53 protein synthesis, we treated TSC1-/- MEFs with MG132 in the presence or absence of glucose. p53 synthesis was measure indirectly by observing its rate of accumulation in the absence of degradation. Glucose starvation slightly decreased the rate of p53 accumulation (Fig 3C). Taken together, this suggests that energy starvation induces p53 accumulation in TSC1-/- MEFs by increasing p53 stability, but not synthesis.

Inhibition of mTOR decreases p53 synthesis without increasing degradation

mTOR inhibition decreases p53 levels, which can be due to decreased synthesis, increased degradation, or a combination of both. To distinguish between these possibilities, we first tested the effect of mTOR activity on p53 phosphorylation during glucose starvation. p53 was accumulated by glucose starvation in TSC1-/- MEFs, and further synthesis blocked by cycloheximide. Not only did rapamycin have no effect on p53 Ser15 phosphorylation, but rapamycin also did not have a significant effect p53 half-life (Fig 4A). In comparison, thirty minutes of rapamycin treatment is sufficient to completely eliminate mTOR-dependent phosphorylation of ribosomal S6 kinase 1 (S6K), a direct downstream target (data not shown). This suggested that mTOR is not responsible for p53 Ser15 phosphorylation and indicates that rapamycin does not destabilize p53 after prolonged glucose starvation.

To more directly test the effect of rapamycin on p53 stability, a ^{35}S-pulse-chase was used to determine p53 stability. In glucose containing media, TSC1-/- MEFs were labeled with ^{35}S-methionine, and then it was chased with cold methionine. Consistent with the results observed by cycloheximide treatment in the absence of glucose, rapamycin also did not significantly reduce the half-life of p53 in the presence of glucose (Fig 4B). The addition of excess

methionine during the cold chase also had no effect on mTOR activity, as assayed by S6K1 phosphorylation (data not shown). Together this suggests mTOR inhibition does not stimulate p53 degradation; therefore, the protective role of rapamycin during glucose starvation is not due to destabilization of p53.

To compare the effects of mTOR activity on p53 synthesis, MG132 was used to block degradation in both TSC1-/- and TSC1+/+ MEFs. p53 accumulation was determined both in the presence and absence of glucose. In the TSC1+/+ MEFs, p53 synthesis was inhibited by glucose starvation. In contrast, in the TSC1-/- MEFs, p53 synthesis continues despite the absence of glucose (Fig 5A). In other words, when the mTOR pathway can be shutdown by energy starvation, p53 synthesis is abated; however, when glucose starvation cannot shut down mTOR, p53 synthesis remains unaffected.

To demonstrate that inhibition of mTOR in the TSC1-/- MEFs can indeed reduce the accumulation of p53, rapamycin was used to inhibit mTOR before the addition of MG132. Pretreatment with rapamycin decreased the rate of p53 accumulation, which suggests that rapamycin indeed reduces p53 synthesis. Furthermore, whether glucose was present in the media had no effect on the rapamycin induced reduction in p53 (Fig 5B). Therefore, mTOR activity seems to be critical for regulating p53 synthesis.

Together, inhibition of mTOR decreases p53 synthesis, but does not affect p53 stability. This suggested that the robust activation of p53 by glucose starvation in the TSC1-/- MEFs was due to unabated p53 synthesis by constitutive mTOR activation. Conversely, the lack of p53 response in the TSC1+/+ MEFs could be explained by inactivation of mTOR by AMPK-dependent phosphorylation of TSC2, which leads to inhibition of p53 synthesis (Inoki et al, 2003).

mTOR regulates the association of p53 mRNA with polysomes

mTOR plays a role in the regulation of both transcription and translation; therefore, to clarify the mechanism by which mTOR affects p53 synthesis, both p53 transcription and translation were examined. In order to determine the effects of mTOR inhibition on TP53 transcription, Quantitative RT-PCR (qRT-PCR) was used to determine p53 mRNA level. After glucose starvation or rapamycin treatment, the level of p53 mRNA was determined and normalized to either actin mRNA or hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA (Fig 5C). Our data indicate that neither rapamycin nor glucose starvation significantly changed p53 mRNA levels.

To examine the effect of mTOR on p53 translation, polysome fractionation was used to determine the fraction of p53 mRNA being actively translated. Lysates were fractionated in a sucrose gradient, and mRNA was collected and analyzed by qRT-PCR to determine the relative distribution of the mRNA. In the untreated TSC1+/+ MEFs, the p53 mRNA was predominately associated with the polysome fractions (Fig 5D). However, rapamycin decreased the percentage of p53 mRNA in the polysome associated fractions and increased the percentage of p53 mRNA in the non-polysome fractions. This shift in p53 mRNA indicated that rapamycin treatment was able to decrease the fraction of p53 mRNA being actively translated (Fig 5D). However, the decrease in p53 translation was not specific to p53 because rapamycin also affected the percentage of polysome associated mRNA for other genes (data not shown). Together, the lack of change in total p53 mRNA and the shift of p53 mRNA away from the polysome by rapamycin suggests that regulation of p53 protein levels by mTOR activation is primarily due to increased translation.

Energy starvation triggers apoptosis by intrinsic pathway in TSC cells

p53 is a potent activator of the intrinsic apoptotic pathway; however, energy starvation can induce cell death via both necrosis or apoptosis. To confirm that induction of p53 was

triggering apoptosis in the TSC1-/- MEFs, Annexin V/Propidium Iodide (PI) double staining was measured by FACS analysis. TSC1-/- MEFs were glucose starved both in the presence and absence of rapamycin. FACS analysis demonstrated that the dying cells induced by glucose starvation were predominately stained by Annexin V and not PI; thus, demonstrating death was predominately apoptotic (Fig 6A). Furthermore, consistent with the protection against gross cell death (Fig 1A Left) and inhibition of p53 synthesis (Fig 2A) seen earlier, rapamycin decreased apoptosis in response to glucose starvation.

To show that p53 was important for inducing apoptosis, FACS analysis was also performed on TSC2-/- p53-/- and TSC2+/+ p53-/- MEFs. Like what was seen by gross visualization (Fig 1B Right) both cell types were equal resistant to glucose starvation (Fig 6B). To exclude the possibility that the TSC2-/- MEFs had a delayed apoptotic response, Annexin V/PI doubling staining was also done after 24 hours of glucose starvation. Again, the TSC2-/- p53-/- cells did not show enhanced sensitivity to glucose starvation (data not shown). In comparison, increase sensitivity to glucose starvation in the TSC1-/- MEFs was readily apparent at 12 hours (Fig 6A). Together, this data suggests that p53 triggers the apoptosis induced by glucose starvation, when mTOR is misregulated.

To further confirm that the intrinsic pathway was activated by glucose starvation, caspase activation was assayed by immunoblot. In the TSC1-/- MEFs, glucose starvation induced the intrinsic death pathways as seen by cleavage of Caspases 9 and 12 (Fig 6C). Furthermore, the executioner Caspase 3 was also activated. In contrast, glucose starvation did not activate Caspase 3, 9, or 12 in the TSC1+/+ MEFs.

To demonstrate that rapamycin also prevented the induction of Caspase cleavage in the TSC1-/- MEFs, mTOR was inhibited by rapamycin during glucose starvation. When TSC1-/- MEFs were rescued with rapamycin, cleavage of Caspases 12 and 9 did not occur; thusly, the intrinsic apoptotic pathway was not activated. Furthermore, glucose starvation had no effect on Caspase 8 (Fig 6D). Taken together, glucose starvation of TSC1-/- MEFs induces apoptosis consistent with the observed changes in p53 activation.

p53 accumulation associated with energy stress in angiomyolipomas

To determine whether our model of regulation of p53 by mTOR was also reflected in vivo, angiomyolipomas were stained by immunohistochemistry. Angiomyolipomas are benign tumors consisting of smooth muscle cells, adipose tissue, and blood vessels of which both the stromal cells and the vasculature demonstrate loss of hetrozygosity for either TSC1 or TSC2, and thusly, mTOR activation(Karbowniczek et al, 2003). Like what was seen in the TSC1-/- MEFs, both sporadic and TSC disease-associated angiomyolipomas showed high levels of p53 and VEGF. It has been shown that VEGF expression can be induced by either hypoxia or loss of TSC (Brugarolas et al, 2003, El-Hashemite et al, 2003); therefore, VEGF staining may indicate areas of energy stress or TSC loss. In patient 774, which is a sporadic angiomyolipoma, both tumor and normal tissue can be compared (Fig 7A). Patients with sporadic angiomyolipomas do not have associated Tuberous Sclerosis disease, but they have Loss of Hetrozygosity of TSC2; therefore, they show upregulated mTOR (Henske et al, 1995). In normal kidney cells, both VEGF and p53 staining are very low. It is interesting to note that there are small areas of VEGF upregulation, which may reflect areas of energy stress; however, in the absence of mTOR activation, p53 levels are universally low. In comparison, within the angiomyolipoma, both VEGF expression and p53 levels are correspondingly elevated. Consistently, in patient 663, which has a TSC-associated angiomyolipoma, both VEGF and p53 are elevated (Fig 7B). Furthermore, the distribution of p53 and VEGF upregulation are also strikingly similar. Together, co-elevation of p53 and VEGF in angiomyolipomas and the lack of elevation of p53 in normal tissue may suggest that loss of TSC1/2 may also contribute to p53 accumulation during energy stress in vivo.

Figure Legend

Fig 1. Dysregulation of mTOR activation sensitizes cells to p53 dependent insults.
(A) TSC1-/- MEFs challenged with glucose starvation (15 hrs) were more prone to death, which was protected against by rapamycin treatment. RNAi knockdown of p53 decreases sensitivity to glucose starvation (15 hrs) in TSC1-/- MEFs. (B) LEF TSC2-/- cells were sensitized to glucose starvation (36 hrs), and both rapamycin and add back of TSC2 eliminated sensitivity. TSC2-/- p53-/- and TSC2+/+ p53-/- MEFs are resistant to glucose starvation (15 hrs). (C) TSC1-/- MEFs are more sensitive to MMS treatment (50 μg/mL, 8 hrs), and rapamycin pretreatment is protective (24 hrs). Infection of HEK293 cells with Rheb L64Q increases sensitivity to MMS treatment (25 μg/mL, 8 hrs). Pretreatment of HEK293 Rheb L64Q cells with rapamycin (24 hrs) protects cells against cell death.

Fig 2. mTOR activation enhances p53 phosphorylation and accumulation.
(A) Glucose starvation time course showed both p53 Ser15 phosphorylation and p53 accumulation in TSC1-/- MEFs, which was reversed by rapamycin (R) and compound C (C, 10 μM); however, this was not seen in TSC1+/+ MEFs. Etoposide treatment (6 μg/mL, 6 hours) induced p53 in both TSC1-/- and TSC1+/+ MEFs. (B) Glucose starvation induced p53 Ser15 phosphorylation and p53 accumulation in TSC2-/- LEFs, which was reverse by rapamycin treatment; however, TSC2+ LEFs did not show induction of p53. (C) Glucose starvation of TSC1-/- MEFs also increased phosphorylation on p53 Ser6, Ser9, Ser20, and Ser392, which was reverse by rapamycin. However, TSC1+/+ MEFs did not show p53 phosphorylation during glucose starvation. (D) p53 phosphorylation and accumulation was stimulated by etoposide. Concurrent rapamycin treatment decreased p53 protein levels, and detected p53 Ser15 phosphorylation. Rapamycin treatment alone also decreased basal p53 levels.

Fig 3. Stabilization of p53 during glucose starvation is due to AMPK.
(A) p53 was stabilized by glucose starvation in TSC1-/- MEFs, and further synthesis was blocked by cycloheximide (50 ng/mL). Reintroduction of glucose 30 minutes before cycloheximide treatment decreased p53 stability. (B) p53 was accumulated with glucose starvation in TSC1-/- MEFs, and degradation was blocked by MG132 (20 μM). Compound C decreased phosphorylation on p53 Ser15, when degradation of p53 was blocked. (C) Degradation of p53 was blocked by MG132 in TSC1-/- MEFs. Glucose starvation did not increase p53 synthesis.

Fig 4. Rapamycin does not affect p53 stability or phosphorylation.
(A) p53 was stabilized by glucose starvation in TSC1-/- MEFs, and further synthesis was blocked by cycloheximide (50 ng/mL). Addition of rapamycin 30 minutes before cycloheximide treatment did not affect p53 stability or phosphorylation on Ser15. (B) ^{35}S Pulse-chase both in the presence and absence of rapamycin in glucose-rich media of TSC1-/- MEFs. Rapamycin did not enhance the degradation of p53.

Fig 5. Inhibition of mTOR decreases p53 synthesis.
(A) p53 degradation was blocked by MG132. Accumulation of p53 was examined under various conditions in TSC1-/- and TSC1+/+ MEFs. In TSC1-/- MEFs, glucose starvation is unable to shut down p53 synthesis. In TSC1+/+ MEFs, glucose starvation decreases the rate of p53 synthesis. (B) TSC1-/- MEFs were pretreated with rapamycin for 6 hours prior to MG132 treatment. Accumulation of p53 was decreased by rapamycin pretreatment regardless of whether glucose was present. (C) p53 mRNA was normalized to either Actin mRNA or HPRT mRNA in TSC1-/- and TSC1+/+ MEFs. Neither glucose starvation (-G, 6 hrs) nor rapamycin (+R, 6 hrs)

treatment had significant effects on p53 mRNA level. (D) p53 mRNA was fractionated over a sucrose gradient in WT MEFs to examine the p53 mRNA association with polysomes. Fractions 8-12 represent polysome associated fractions. Rapamycin decreased polysome association of p53 mRNA.

Fig 6. Energy starvation triggers apoptosis via intrinsic pathway in TSC cells.
(A) Annexin V (X-axis) / Propidium Iodide (Y-axis) double staining showed glucose starvation (12 hrs) induced cells death predominately through apoptosis as opposed to necrosis in TSC1-/- MEFs. Early apoptotic cells can be stained by Annexin V, which binds to phosphotidyl-serines normally found in the inner-aspect of the cell membrane, but can be found on the outer-aspect of the cell membrane in apoptotic cells. On the other hand, during early apoptosis, PI, which stains DNA, and is excluded from the nucleus, so staining does not occur. During necrosis and late apoptosis, membrane integrity is compromised, and cells are stained by both Annexin V and PI. (B) FACS analysis showed that TSC2-/- p53-/- and TSC2+/+ p53-/- MEFs were equally resistant to energy starvation (15 hrs). (C) Glucose starvation induced cleavage of Caspases 12, 9, and 3 in TSC1-/- MEFs but not in TSC1+/+ MEFs. (D) Rapamycin treatment during glucose starvation prevented Caspase 12 and 9 cleavage. Caspase 8 was not cleaved by glucose starvation.

Fig 7. Energy stress in angiomyolipomas is associated with p53 upregulation and model of p53 activation by energy starvation in TSC-/- cells.
(A) Tissues from both normal kidney and sporadically arising angiomyolipomas were stained for p53 and VEGF. Normal tissue showed little upregulation of either p53 or VEGF, while in the angiomyolipoma, both p53 and VEGF staining were dramatically increased. (B) Tissues from TSC patient derived angiomyolipomas were stained for p53 and VEGF. Both p53 and VEGF were correspondingly increased. (C) Model for negative regulation of p53 by mTOR to promote survival during stress. When the mTOR pathway is intact, AMPK activation down regulates p53 synthesis via the mTOR pathway and stabilizes p53 via phosphorylation. However, in the absence of TSC, p53 synthesis cannot be down regulated; therefore, when AMPK stabilizes p53, p53 is greatly elevated, and apoptosis is induced.

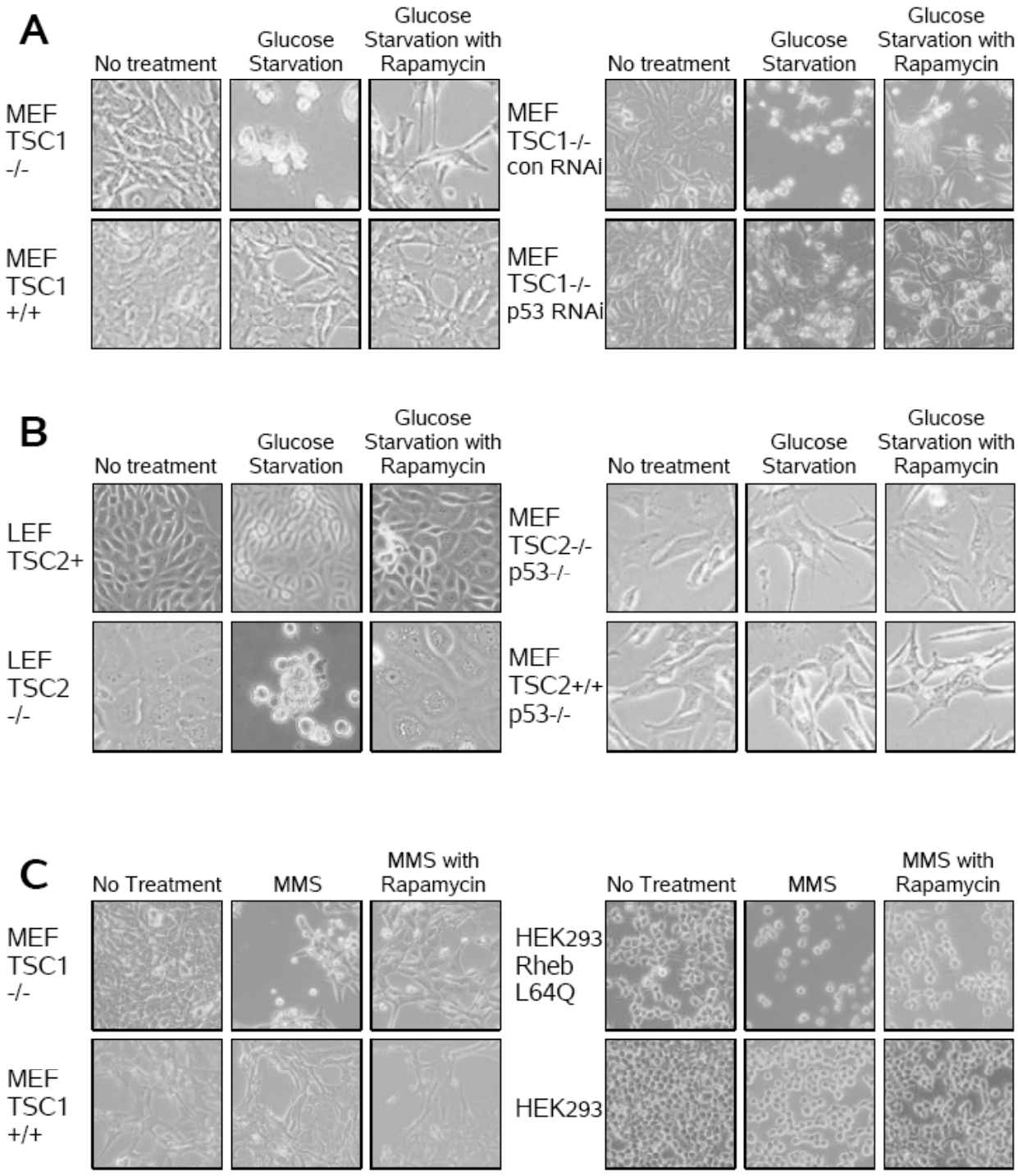
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Figure 1

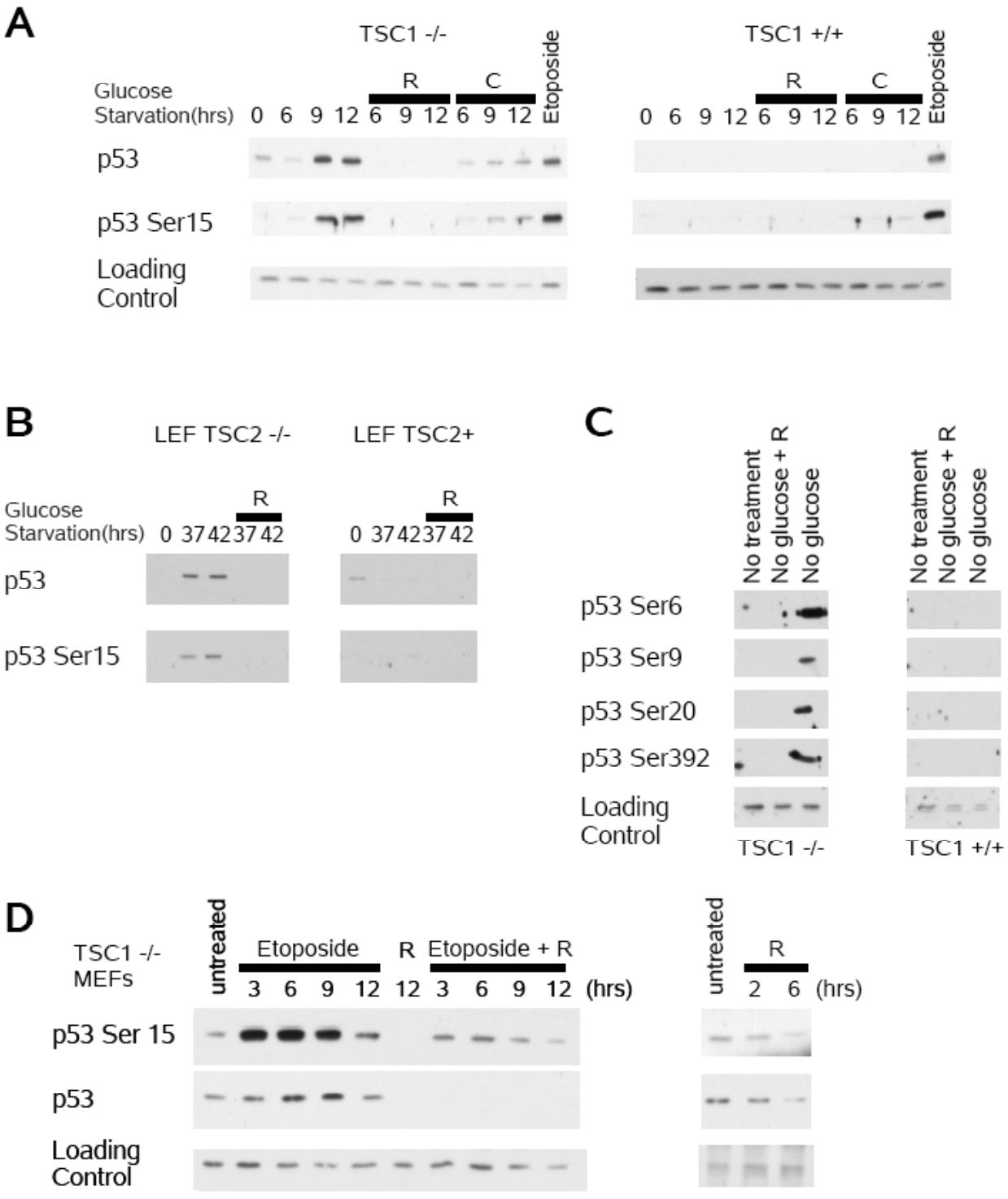
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Figure 2

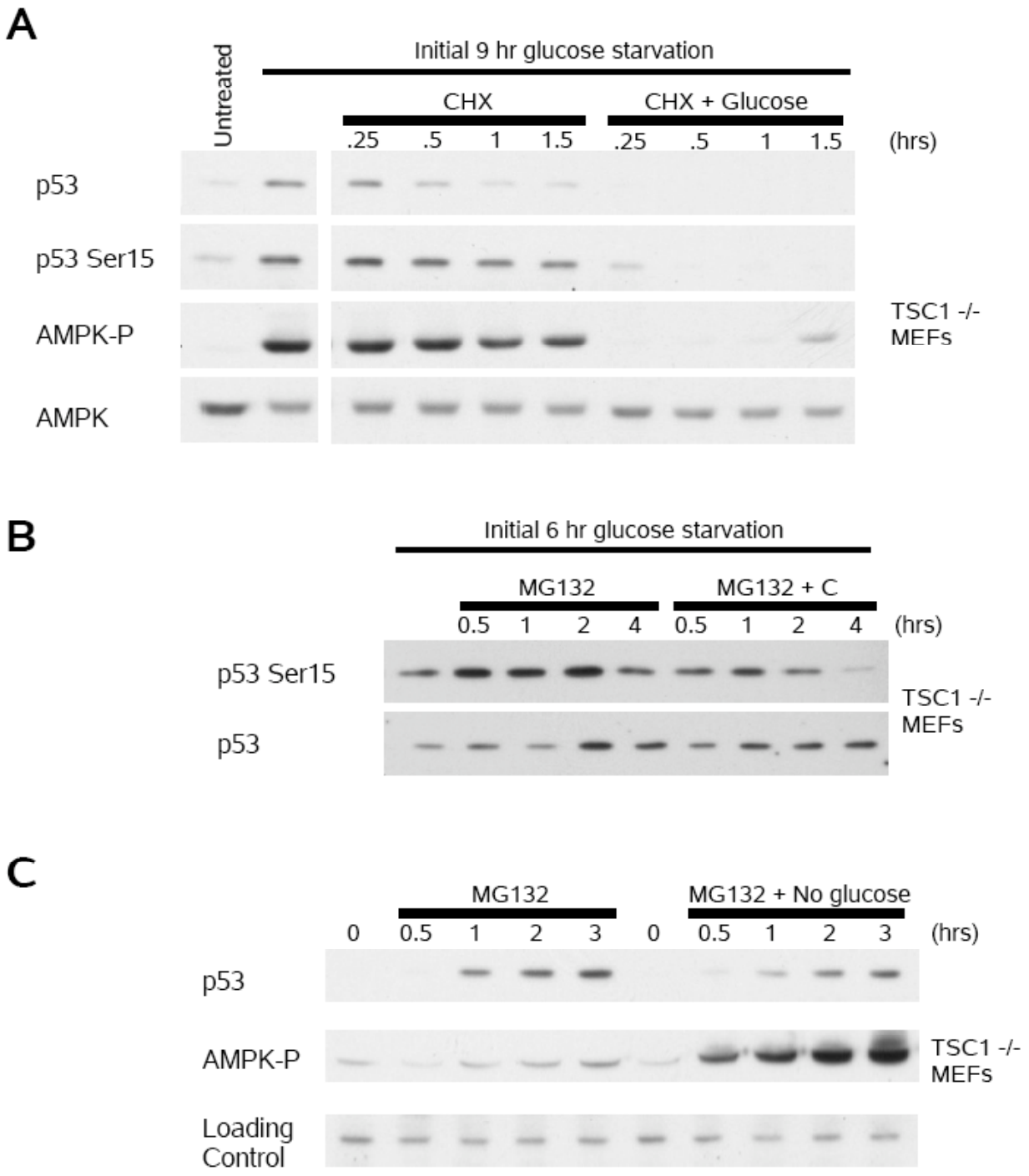
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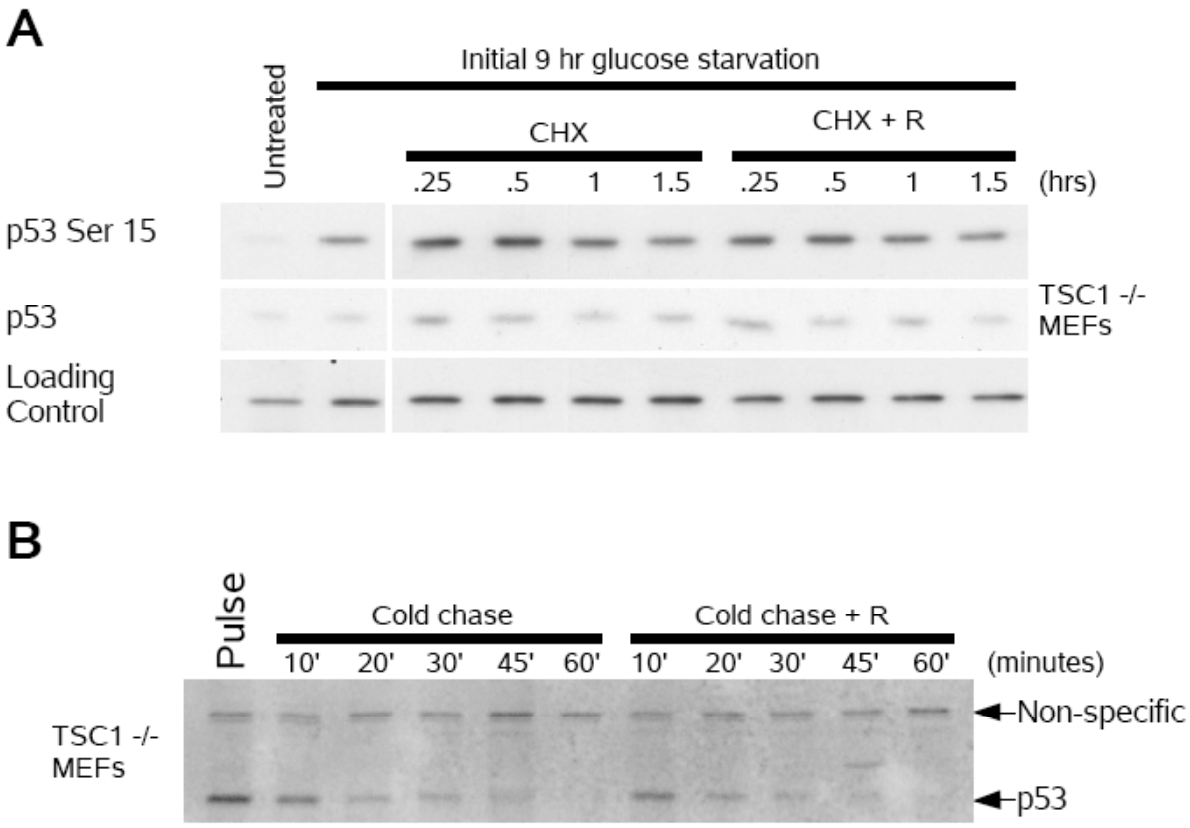
Figure 3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Cold chase + R
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(minutes)
TSC1 -/- MEFs
Non-specific
p53

Figure 4

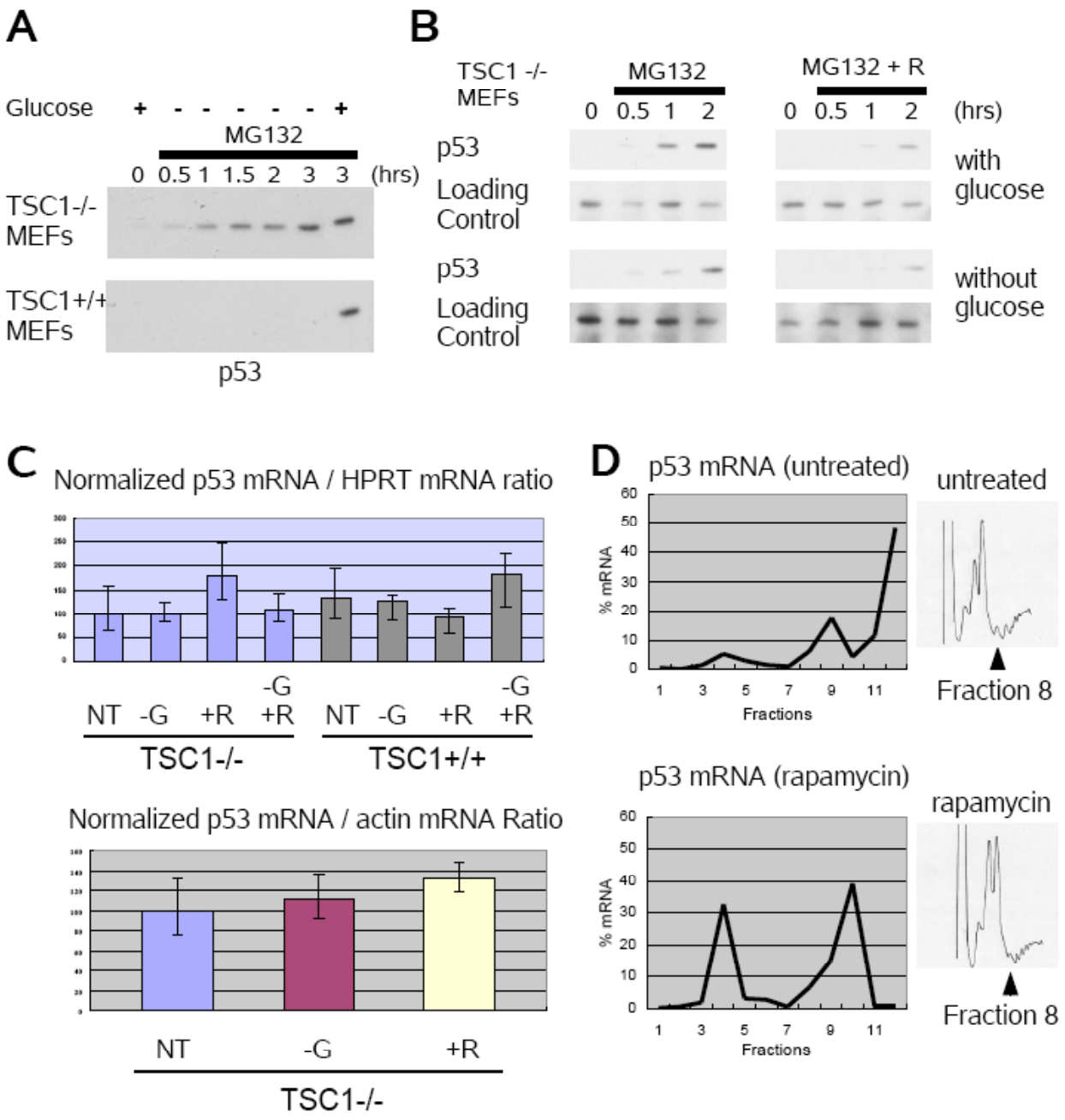
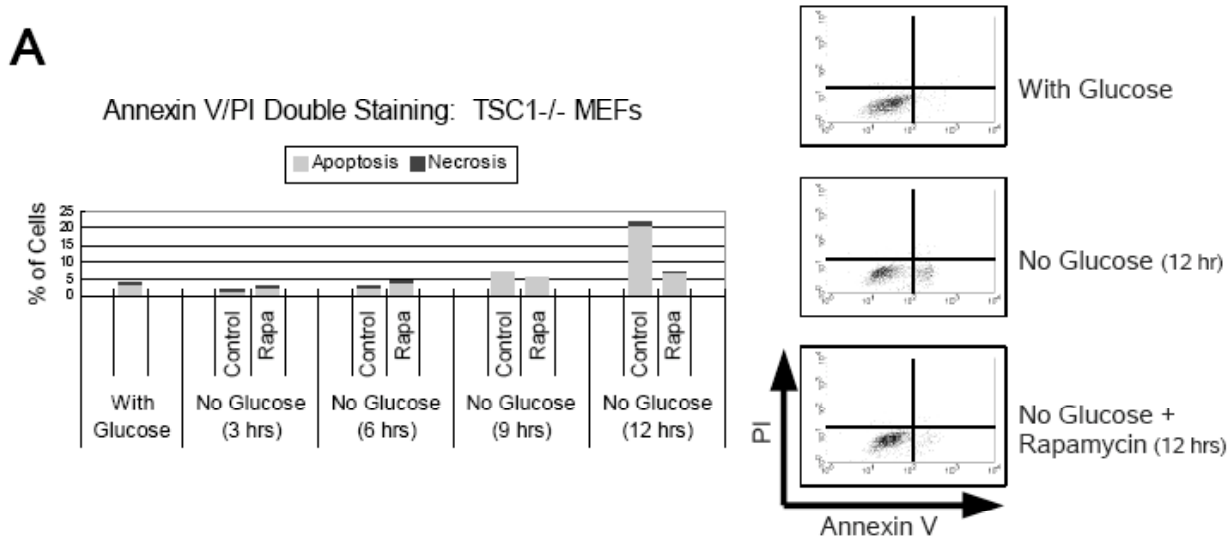
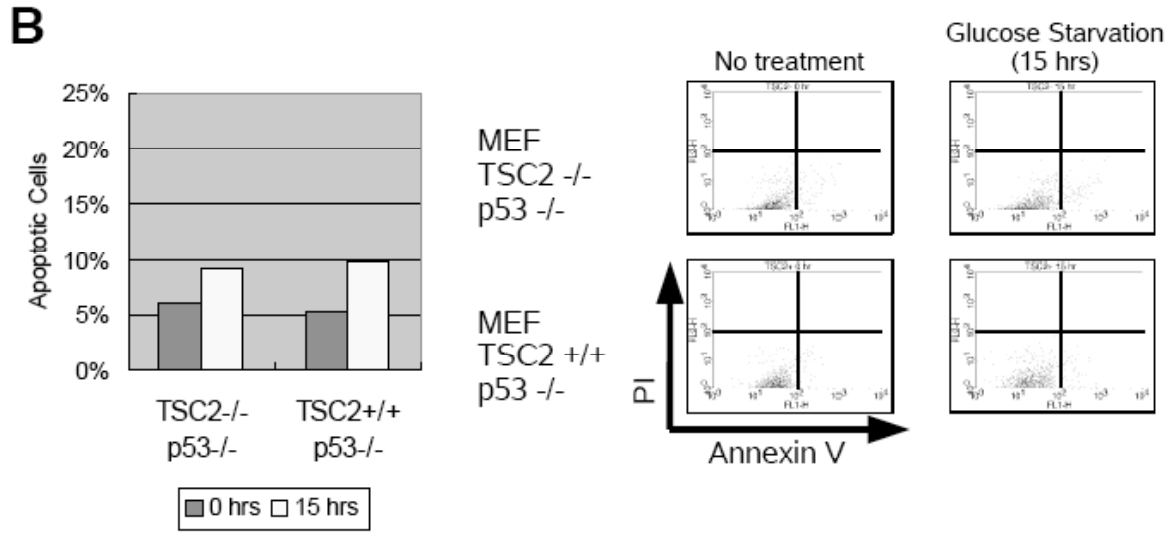
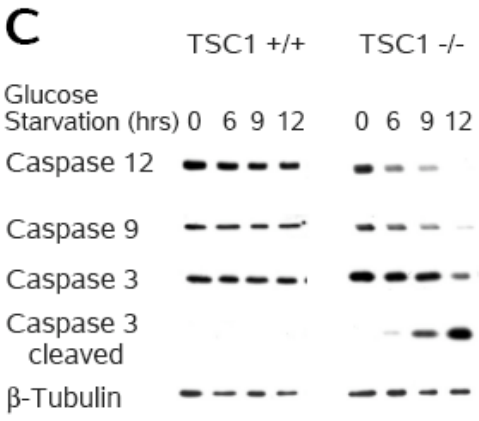

A
Glucose + - - - - - +
MG132
0 0.5 1 1.5 2 3 3 (hrs)
TSC1-/- MEFs
TSC1+/+ MEFs
p53
B
TSC1 -/- MEFs
MG132
MG132 + R
0 0.5 1 2
0 0.5 1 2 (hrs)
p53
Loading Control
with glucose
p53
Loading Control
without glucose
C
Normalized p53 mRNA / HPRT mRNA ratio
NT -G +R -G +R
NT -G +R -G +R
TSC1-/-
TSC1+/+
Normalized p53 mRNA / actin mRNA Ratio
NT -G +R
TSC1-/-
D
p53 mRNA (untreated)
% mRNA
Fractions
untreated
Fraction 8
p53 mRNA (rapamycin)
% mRNA
Fractions
rapamycin
Fraction 8

Figure 5


A
Annexin V/PI Double Staining: TSC1-/- MEFs
Apoptosis
Necrosis
% of Cells
Control
Rapa
With Glucose
No Glucose (3 hrs)
No Glucose (6 hrs)
No Glucose (9 hrs)
No Glucose (12 hrs)
With Glucose
No Glucose (12 hr)
No Glucose + Rapamycin (12 hrs)
PI
Annexin V


B
Apoptotic Cells
25%
20%
15%
10%
5%
0%
TSC2-/- p53-/-
TSC2+/+ p53-/-
0 hrs
15 hrs
MEF TSC2 -/- p53 -/-
MEF TSC2 +/+ p53 -/-
No treatment
Glucose Starvation (15 hrs)
PI
Annexin V


C
TSC1 +/+
TSC1 -/-
Glucose Starvation (hrs) 0 6 9 12 0 6 9 12
Caspase 12
Caspase 9
Caspase 3
Caspase 3 cleaved
β-Tubulin

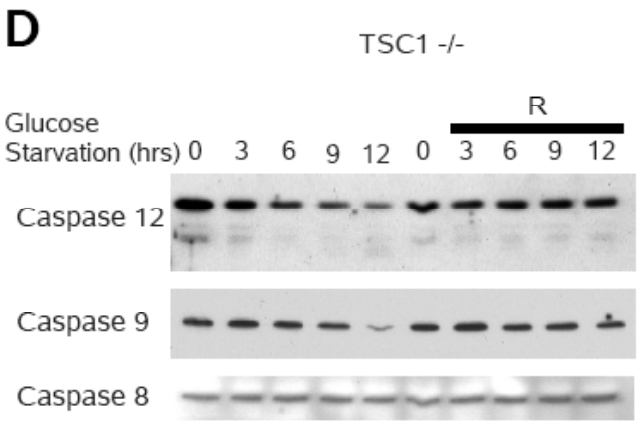

D
TSC1 -/-
R
Glucose Starvation (hrs) 0 3 6 9 12 0 3 6 9 12
Caspase 12
Caspase 9
Caspase 8

Figure 6

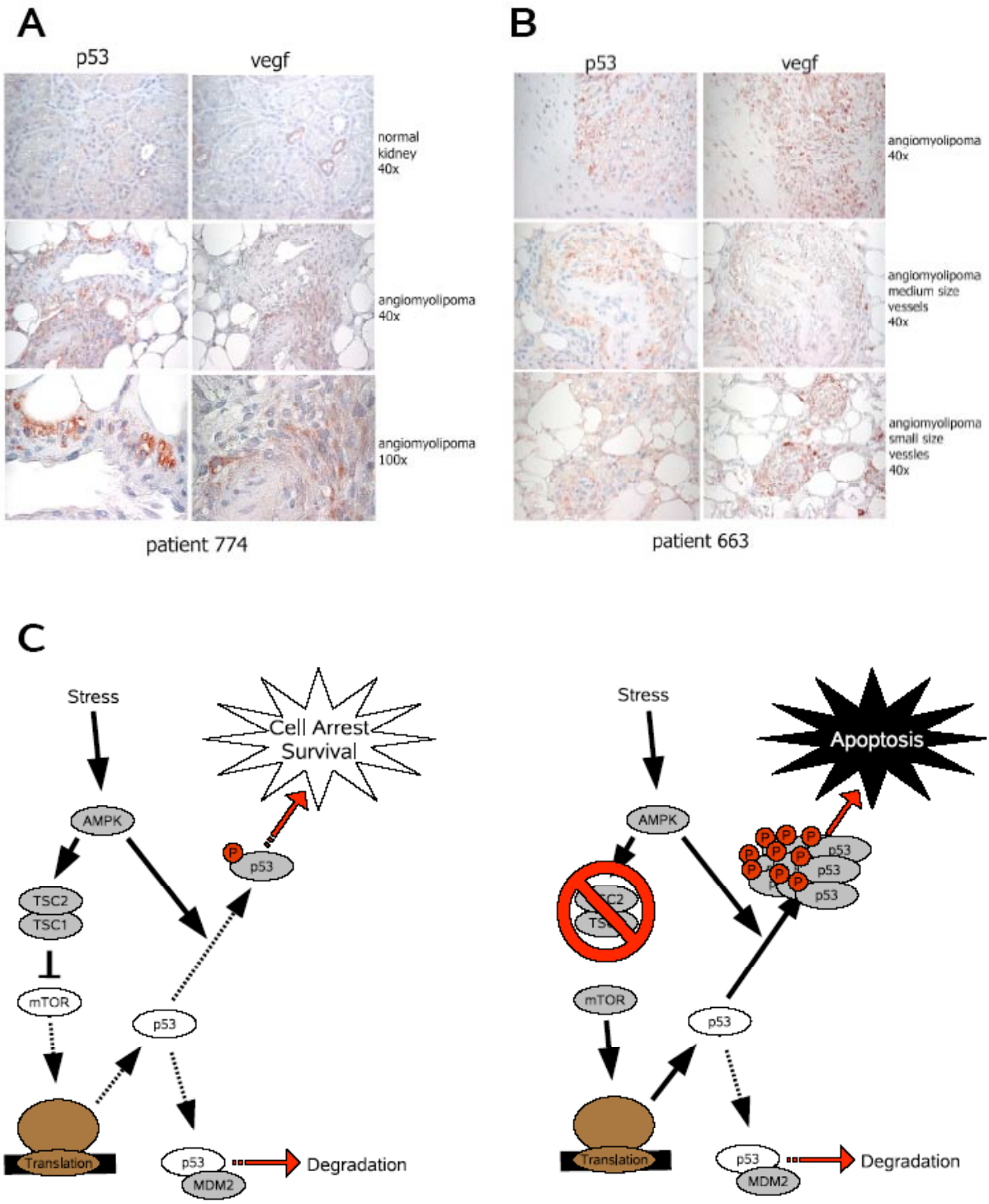

A
p53
vegf
normal kidney 40x
angiomyolipoma 40x
angiomyolipoma 100x
patient 774
B
p53
vegf
angiomyolipoma 40x
angiomyolipoma medium size vessels 40x
angiomyolipoma small size vessles 40x
patient 663
C
Stress
Cell Arrest Survival
AMPK
TSC2
TSC1
mTOR
p53
Translation
p53
MDM2
Degradation
Stress
Apoptosis
AMPK
p53
mTOR
p53
Translation
p53
MDM2
Degradation

Figure 7

KEY RESEARCH ACCOMPLISHMENTS

1. We showed that p53 protein level is dramatically induced by energy starvation in TSC1-/- or TSC2-/- cells.
2. We showed that p53 is required for energy starvation induced apoptosis in TSC2-/- cells.
3. We demonstrated that p53 is phosphorylated on S15, S20, S392 by energy starvation.
4. We showed that AMPK contributes to p53 stabilization.
5. We showed that mTOR is involved in p53 translation and p53 mRNA association with polysome.
6. We showed that p53 is elevated in angiomyolipomas.

REPORTABLE OUTCOMES

Chung-Han Lee, Ken Inoki, Magdalena Karbowniczek, Emmanuel Petroulakis, Nahum Sonenberg, Elizabeth Petri Henske, and Kun-Liang Guan. Activation of mTOR sensitizes cells to p53 dependent apoptosis. (manuscript submitted).

CONCLUSION

We have successfully accomplished all the goals outlined in the original proposal. We have demonstrated that p53 accumulation is essential for apoptosis in TSC mutant cells in response to energy starvation. We have shown that mTOR regulates p53 synthesis, and continued synthesis of p53 by mTOR activation sensitizes cells to p53 activators. Aberrant mTOR activation leads to increased sensitivity to both DNA damage and energy starvation. Interestingly, energy starvation can also lead to AMPK activation, which in turn phosphorylates and stabilizes p53. The dramatic accumulation of p53 is due to the combined effect of stabilization by AMPK and continued synthesis by elevated mTOR. Consequently, cells, such as TSC tumor cells, which cannot inhibit mTOR are faced with runaway p53 activation under energy starvation. Therefore, our results explain the sensitivity of TSC mutant cells to energy starvation.

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